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Interaction of rotavirus with human peripheral blood mononuclear cells: Plasmacytoid dendritic cells play a role in stimulating memory rotavirus specific T cells *in vitro*

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Abstract

We studied the interaction of RV with human peripheral blood mononuclear cells (PBMC) from adult volunteers. After exposure of PBMC to rhesus RV (RRV), T and B lymphocytes, NK cells, monocytes, and myeloid and plasmacytoid dendritic cells expressed RV non-structural proteins, at variable levels. Expression of these RV proteins was abolished if infection was done in the presence of anti-VP7 neutralizing antibodies or 10% autologous serum. Supernatants of RRV exposed PBMC contained TNF-α, IL-6, IFN-α, IFN-γ, IL-2 and IL-10. Plasmacytoid DC were found to be the main source of IFN-α production, and in their absence the production of IFN-γ and the frequency of RV specific T cells that secrete IFN-γ diminished. Finally, we could not detect RV-antigen associated with the PBMC or expression of RV non-structural proteins in PBMC of acutely RV-infected children. Thus, although PBMC are susceptible to the initial steps of RV infection, most PBMC of children with RV-gastroenteritis are not infected.
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Keywords: Rotavirus; Plasmacytoid dendritic cells; PBMC; Interferon gamma; Interferon alpha; Children; Memory T lymphocytes

Introduction

Rotavirus (RV) is the single most important etiological agent of severe gastroenteritis (GE) in children (Parashar et al., 2003, 2006). Although two new RV vaccines have been recently licensed, improvement of these vaccines and development of future RV vaccines is hampered by our limited knowledge of the mechanisms that mediate immunity against RV in humans (Franco et al., 2006).

The way RV antigen is presented to stimulate naive T cells or re-stimulate memory T cells is poorly understood. Given that RV predominantly infects and replicates in enterocytes, dendritic cells (DC) present in the intestine are probably the initial RV-antigen presenting cells (APCs). However, despite its "enteric nature", RV antigen, dsRNA and infectious particles have been found in blood of children and systemic organs in animals (Azevedo et al., 2005; Blutt and Conner, 2007; Blutt et al., 2003, 2006; Crawford et al., 2006; Fenaux et al., 2006; Fischer et al., 2005; Nakagomi and Nakagomi, 2005; Ray et al., 2006). For this reason, a systemic immune response to RV is expected.

As an initial step to try to understand how RV interacts with systemic APCs, we studied monocyte-derived DC (moDC) exposed to RV (Narvaez et al., 2005). MoDC are considered a model for studying human myeloid DC (mDC). We found that while very low numbers of immature moDC expressed NSP4, up to 46% of mature moDC expressed NSP4, indicating that at least the initial steps of viral infection can occur in moDC. Moreover, our results suggested that although RV can promote the capacity of moDC to prime naive cells to become Th1 cells, it does not seem to be a strong stimulus to make efficient APCs, leaving unclear their role in viral antigen presentation.

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In the present study, we evaluated if peripheral blood mononuclear cells (PBMC) from healthy adults exposed to rhesus RV (RRV) became infected, and the pattern of cytokines secreted by these cells. We also assessed the role of plasmacytoid DC (pDC), in the RRV exposed PBMC cultures, as a source of IFN-α and in stimulating RV-specific memory T cells. Finally, we explored if PBMC from RV-infected children are associated with RV antigen and/or express RV NSPs.

Results

Human PBMC are susceptible to the initial steps of infection with RV

To determine if RVs have the potential to infect PBMC, cells from healthy adult volunteers were exposed to purified RRV (MOI=5) or a Mock control preparation during 0–48 h. We evaluated the presence of the viral protein NSP4 in T, B, NK cells, monocytes, mDC and pDC by flow cytometry, as an indicator of the initial infectious process. Expression of NSP4 was detected 4 h post infection (PI) in all cells analyzed (Fig. 1), with the highest frequencies observed in B cells, monocytes and DC. At 8 h PI, similar percentages of T, B and NK cells expressing NSP4 were observed, while the frequencies of monocytes, mDC and pDC expressing NSP4 tended to decrease (Fig. 2). Additional experiments showed similar levels of NSP4 expression in T ($n=5$), B ($n=8$) and NK ($n=3$) cells exposed to RRV in the form of unpurified tissue culture supernatant (data not shown). Exposure of PBMC to both unpurified RRV and the Mock control preparation (but not with the purified RRV and its control preparation) induced changes in the phenotype of monocytes and DC, precluding their analysis (data not shown). In addition, in 5 experiments the staining pattern of B cells with anti-NSP2, another RV NSP, was similar to that observed with anti-NSP4 (data not shown).

To determine if the initial steps of viral infection could also occur in PBMC with a homologous human RV, PBMC were exposed to partially purified Wa (MOI=5) and a Mock control for 0 to 8 h. NSP4 expression was seen in Wa treated B cells ($n=4$) but not T ($n=5$) or NK cells ($n=3$). The frequency of B cells expressing NSP4 after infection with Wa was from 3.5 to 12 times lower than after infection with RRV (Fig. 3B). In addition, the percentages of expression of NSP4 and NSP2 in B cells infected with Wa were similar (data not shown). In conclusion, several subsets of PBMC, especially B cells, monocytes and DC, are susceptible to the initial steps of RV infection.

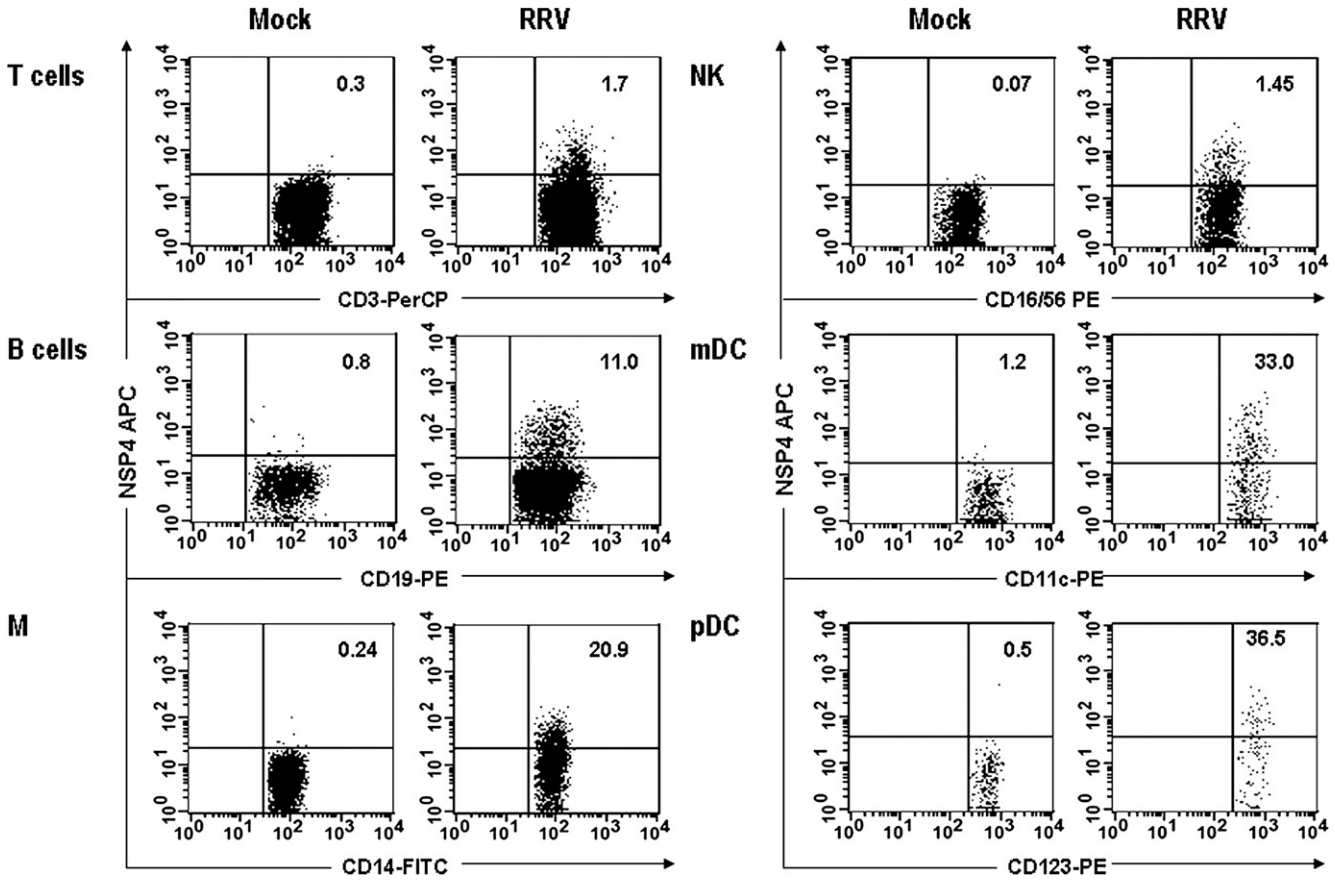

Fig. 1. Expression of RV NSP4 by RRV exposed human PBMC. Intracellular NSP4 expression was observed in T, B, NK cells, monocytes (M) and DC after exposure of human PBMC to purified RRV (MOI=5) during 4 h. The number in the quadrant represents the percentage of NSP4+ cells for each cell subset. Data are representative of 2–4 independent experiments.

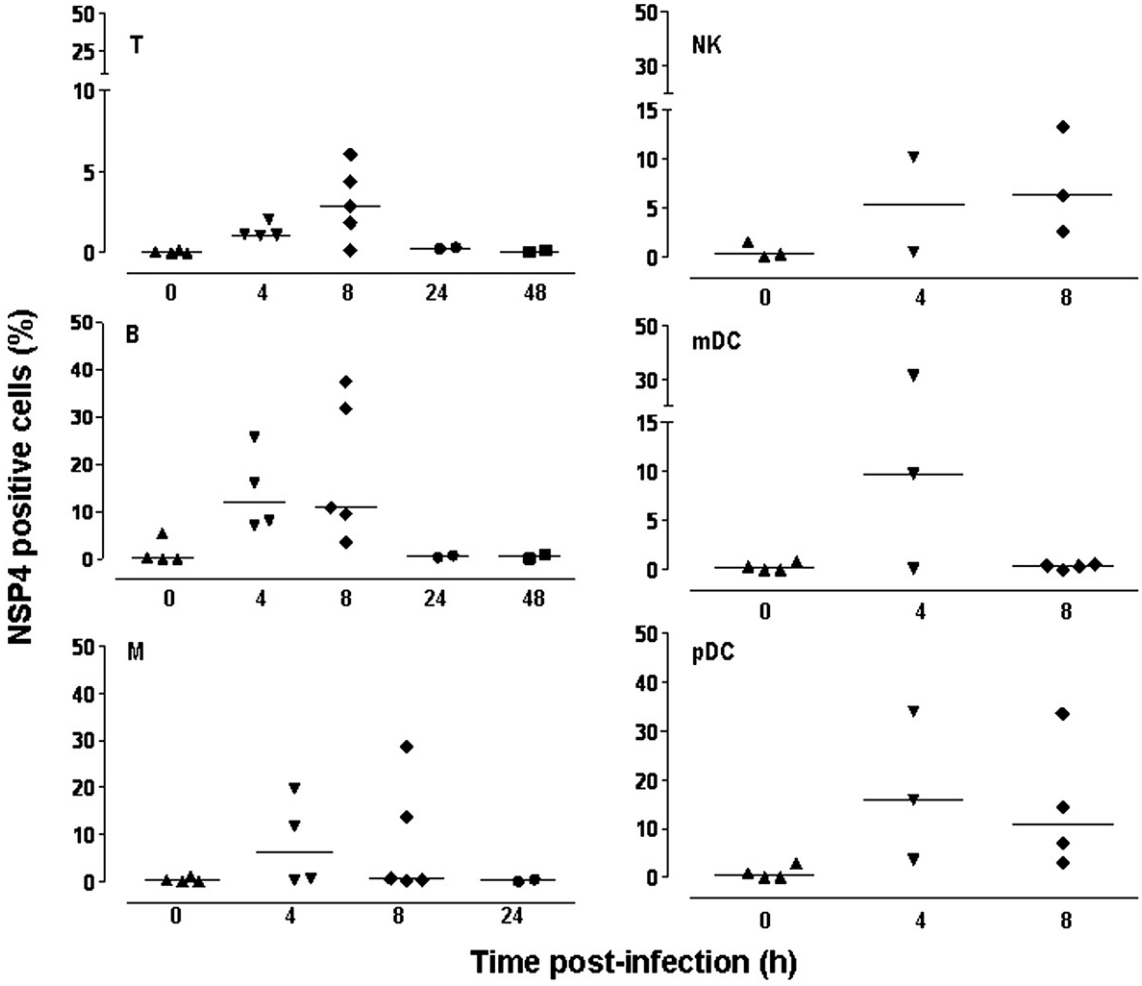

Fig. 2. Differential expression of RV NSP4 among different human PBMC subpopulations at different times PI. PBMC from healthy adult volunteers (n=2–5) were exposed to purified RRV (MOI=5) during 0–48 h. Shown are net values and medians for the % NSP4+ cells in each subset. Less than 1.1% background cells expressed NSP4 in Mock treated control PBMC.

Neutralizing antibodies to VP7 and autologous serum block the expression of NSPs by PBMC

To determine if neutralizing antibodies can block the expression of NSPs by PBMC exposed to RV, cells were infected with purified RRV for 4 h in the presence of the neutralizing anti-VP7 monoclonal antibody (MAb) 159 or an isotype control MAb. A decrease in viral NSP4 protein expression was observed in all cellular subpopulations analyzed (Fig. 3A). Similarly, although to a lesser degree, the pretreatment of PBMC with 10% autologous serum showed inhibition of RRV infection (Fig. 3A). However, we observed that the inhibition induced by autologous serum was somewhat variable between volunteers studied (compare Fig. 3A and B performed with cells from different volunteers), this is probably due to differences in their levels of RV neutralizing antibodies. Similar results were observed for T, B and NK cells exposed to RRV tissue culture supernatant for 8 h (n=3; Fig. 3B, and data not shown). MAb 159 (homotypic for G3, but partially cross-reactive with serotype G1 viruses at high doses) induced limited inhibition of NSP4 expression by B cells exposed to Wa, while MAb 2C9 (homotypic for G1 viruses) and 10% autologous serum drastically reduced the expression of NSP4 in B cells exposed to this same virus (Fig. 3B). Thus, the initial steps of infection of PBMC by RV are efficiently neutralized by human serum and classical neutralizing anti-VP7 antibodies.

PBMC exposed to RV initially secrete TNF-α, IL-6, and IFN-α followed by IL-2, IFN-γ and IL-10

To determine the pattern of cytokines secreted by PBMC exposed to RV, we collected supernatants from both Mock and purified RRV treated PBMC cultures and evaluated the presence of: IL-12p70, TNF-α, IL-6, IL-1β, IL-8, IFN-α; IL-2, IFN-γ; IL-4, IL-5, IL-10 and TGF-β. Four hours PI, PBMC secreted significant amounts of IFN-α, TNF-α and IL-6. Forty-eight hours PI TNF-α levels returned to baseline, while IL-6 and IFN-α levels remained constant. IFN-γ secretion was detected by 8 h PI and reached the highest levels by 48 h PI. Low levels of IL-2 and IL-10 were observed at 24 h PI and a modest increase was seen by 48 h PI (Fig. 4). Increases in IL-12p70, IL-

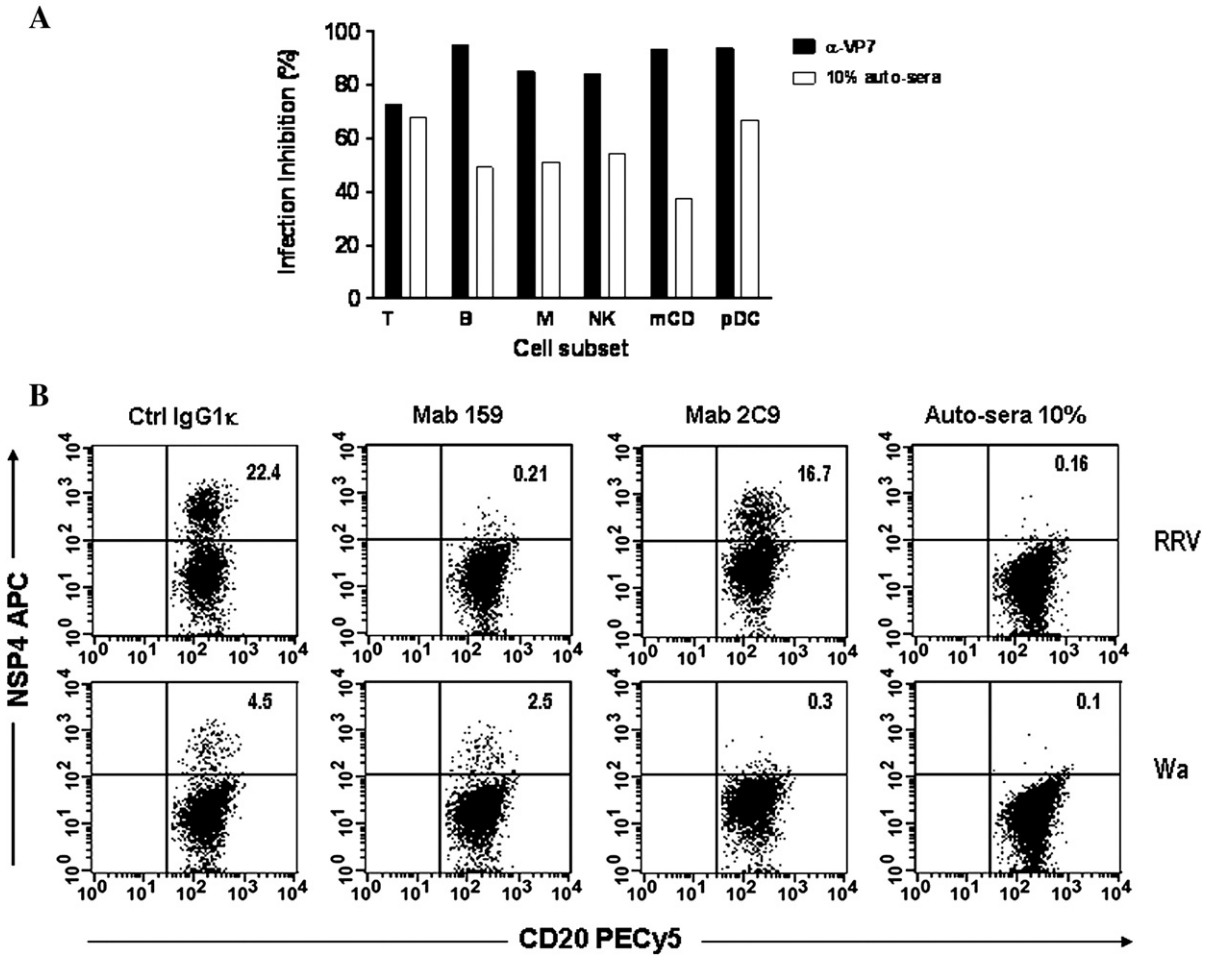

Fig. 3. Anti-VP7 MAbs and autologous serum inhibit early steps of RV infection by PBMC. (A) PBMC from healthy adult volunteers were exposed to purified RRV (MOI=5) for 4 h, in the presence anti-VP7 MAb 159 or 10% autologous serum. Data are expressed as percentage of inhibition compared with cells not exposed to the inhibitors. This result is representative of two independent experiments. (B) PBMC were exposed to either RRV (top row dot plots) or Wa (bottom dot plots) at an MOI of 5 during 8 h in the presence of an isotype control (IgG1κ MAb), anti-VP7 MAbs 159 or 2C9 or 10% autologous serum. The numbers in the upper quadrants are the percentage of B cells expressing NSP4. One of three representative experiments using MAb 159 and autologous serum is shown.

1β, IL-4, IL-5 and active TGB-β were not detected (data not shown). Small increases in IL-8 and latent TGF-β were observed in RRV exposed PBMC, but the significance of these findings are uncertain because of the relatively high levels of these cytokines present in control treated PBMC (data not shown). In the above experiments, PBMC were simultaneously treated with SEB and CpG 2395, as positive controls. As expected, SEB induced pro-inflammatory, Th1 and Th2 cytokines. CpG 2395 induced all pro-inflammatory cytokines, except IL-12p70 and IL-1β; it did not induce detectable levels of IL-2, IFN-γ or Th2 cytokines, and only a very modest secretion of IL-10 (data not shown).

pDC are the principal source of IFN-α produced by PBMC exposed to RV and are associated with the induction of IFN-γ

IFN-α is one of the most important cytokines induced during the course of viral infections (Liu, 2005). Many blood cells can produce IFN-α, but pDCs have been shown to be the principal source (Ito et al., 2006; Liu, 2005; Siegal et al., 1999). To determine if pDC were contributing to the IFN-α produced by RV stimulated PBMC, we stimulated PBMC, depleted or not of pDC, with RRV. Depletion of pDC in these experiments was above 91%. Secretion of IFN-α was reduced by over 95% in pDC-depleted PBMC (Fig. 5A). Importantly, the levels of IFN-γ were also reduced (median=80.6%; range 55.4–96.1%) in these supernatants (Fig. 5B). Levels of IFN-α and IFN-γ were not reduced in control PBMC depleted of B cells or treated with GAM-coated beads (data not shown).

To confirm and extend the previous results, we studied the simultaneous presence of intracellular NSP4 protein and of IFN-α in pDC collected from PBMC exposed to RRV. Most pDC (Fig. 6) neither expressed NSP4 nor secreted IFN-α (range 45–79%; $n=3$). A small fraction (0.6 to 12.9%; $n=3$) of pDC expressed NSP4 and secreted IFN-α. In addition, pDC that secreted IFN-α, but did not express NSP4 (from 4.5 to 19.4%; $n=3$) and pDC that expressed NSP4 but did not secrete IFN-α (from 9.4 to 35.4%; $n=3$) were also observed. Thus, both infected and non-infected pDC seem to be the main source of IFN-α when PBMC are exposed to RV.

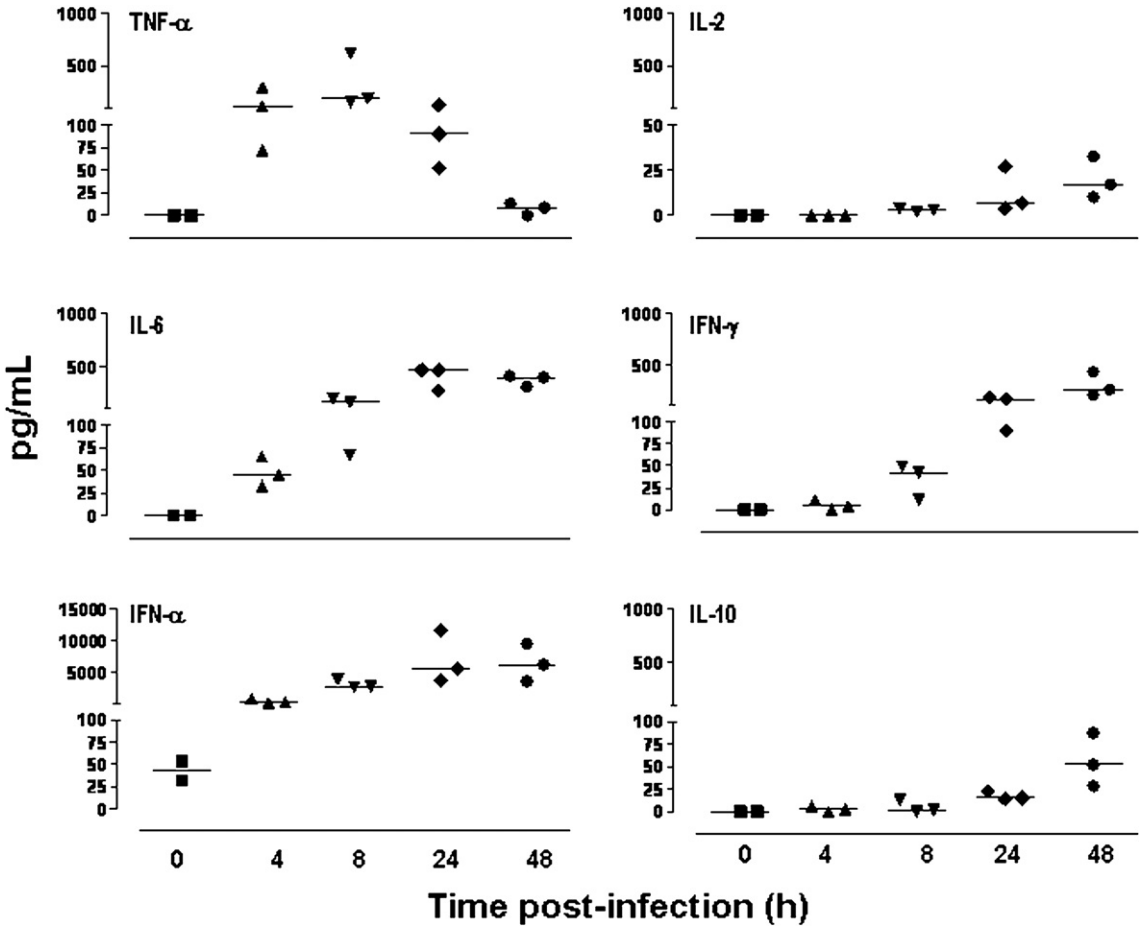

Fig. 4. Cytokines secreted by PBMC exposed to RRV. Supernatants collected from PBMC exposed to purified RRV (MOI=5) for 0–48 h were evaluated by CBA and ELISA for pro-inflammatory, Th1, Th2 and Treg cytokines. Shown are net values and medians from RRV treated cells (n=3).

pDC are necessary to stimulate RV-specific memory T cells

Because depletion of pDC from PBMC also diminished their capacity to secrete IFN-γ (Fig. 5B), we addressed the involvement of circulating pDC in the stimulation of IFN-γ secreting RV-specific memory T cells. For this purpose, we stimulated total and pDC-depleted PBMC with RRV (MOI=5) in a short term (10 h) assay. As shown in Fig. 7, pDC depletion was associated with a significant decrease in the frequency of RV-specific CD4+ (median non-depleted PBMC=0.15%, range=0.05–0.25% *vs.* median depleted PBMC=0.04%, range=0.0–0.09%, n=7, Wilcoxon test p=0.01) and CD8+ (median non-depleted PBMC=0.07%, range=0.01–0.19% *vs.* median depleted PBMC=0.01%, range=0.0–0.16%, n=7, Wilcoxon test p=0.03) T cells that secrete IFN-γ. Control PBMC depleted of B cells or treated with GAM-coated beads did not show reductions in the numbers of RV-specific T cells (data not shown).

PBMC from children with RV GE are not infected with RV

To determine if wild type RV could be infecting or be associated with PBMC *in vivo*, we evaluated RV-antigen in plasma and PBMC of 17 children with acute GE (Table 1). Three groups of children were studied: Children in group I (GI) did not have RV antigen/RNA in feces or plasma (n=2); Group II (GII) children had RV antigen and/or RNA in feces but not in plasma (n=6); and Group III (GIII) children had RV antigen and/or RNA in feces and plasma (n=9). No differences were seen in the time after onset of diarrhea, episodes of vomit or diarrhea per day between children of GII and GIII. The median age of children in GII (11.5 months range: 3–14 months) was significantly lower than that (median 17 months range: 10–20 months) of children in GIII (Mann–Whitney p=0.017), a tendency previously observed (Ray et al., 2006). Only three children, one from GI and two from GII, had evidence of previous exposure to RV, indicated by the presence of circulating RV specific IgA in plasma. Among the RV+ stool samples, the P[8] genotype was the most frequently detected (12/15), while P[6] (2/15) and P[4] (1/15) RV were also identified.

Compatible with previous observations (Fischer et al., 2005; Ray et al., 2006), ELISA ODs for RV-antigen in feces and plasma tended to correlate (Spearman rho: 0.84 p=0.05) for GIII children. We were not able to detect RV-antigen by ELISA in lysates of PBMC from three children from GII and four children from GIII (data not shown). Furthermore, with the flow cytometry assay used above to identify initial steps of infection of adult PBMC *in vitro*, we did not detect intracellular NSP4 in the T cells and B cells (n=16; 2/GI, 6/GII, 8/GIII) or monocytes (n=8;

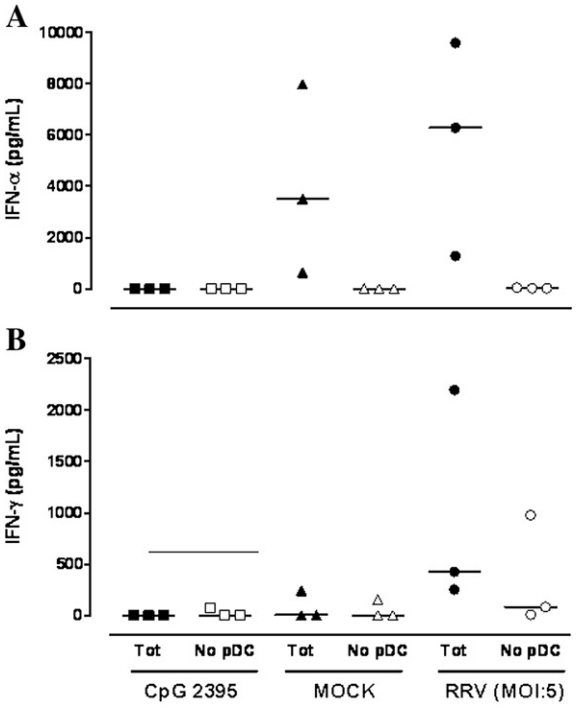

Fig. 5. pDC are the main source of IFN-α in PBMC exposed to RV. (A) pDC depleted PBMC (5×10^6) were exposed to Mock or purified RRV (MOI=5) for 48 h, and supernatants were collected to measure IFN-α by ELISA. (B) IFN-γ levels from the same supernatants were evaluated by CBA. CpG 2395 0.038 μM was used as positive control for IFN-α secretion by pDC (n=3). Individual and median values are shown.

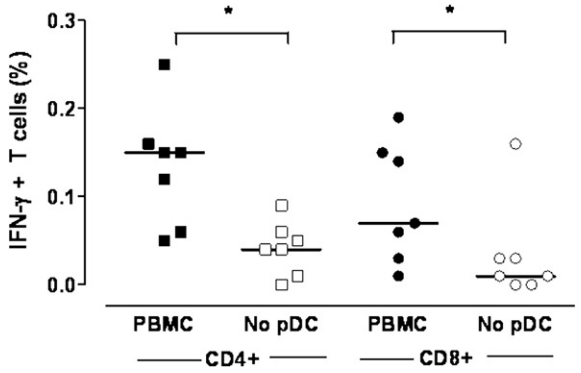

Fig. 7. Human pDC are necessary for efficient stimulation of IFN-γ secreting RV memory T cells. Total, and pDC depleted PBMC (2×10^6/2 ml) were exposed to RRV lysate (MOI=5) or Mock control in the presence of anti-CD28 and anti-CD49d MAbs for 10 h. Brefeldin A was added during the last 5 h. Intracellular IFN-γ was determined in CD69+ activated CD4+ and CD8+ T cells. Bars represent the median (n=7).

2/GI, 1/GII, 5/GIII), NK cells (n=6; 0/GI, 4/GII, 2/GIII), mDC (n=12; 2/GI, 4/GII, 6/GIII) or pDC (n=4; 4/GIII). Staining for intracellular expression of RV-NSP2 was also negative in T cells, B cells and monocytes from 3 children studied simultaneously for NSP4 expression (1/GII and 2/GIII).

In conclusion, we could not detect RV antigen associated with PBMC of children with acute RV infection either by ELISA, or using the same flow cytometry assay with which we could detect infection of adult PBMC using high MOIs of tissue culture adapted RV.

Discussion

We have studied the interaction of RV with PBMC and shown that: (i) RV entered and directed *in vitro* the synthesis of NSP in several PBMC subsets, particularly in B, monocytes cells and DC; (ii) expression of NSPs was blocked by neutralizing antibodies against VP7 and autologous serum; (iii) RRV induced PBMC to initially secrete pro-inflammatory cytokines (IL-6, TNF-α and IFN-α) and subsequently Th1/regulatory cytokines (IFN-γ, IL-2 and IL-10); (iv) the main source of the IFN-α were pDC; (v) the presence of pDC seems critical to stimulate IFN-γ secreting RV-memory T cells, and (vi) RV antigen present in blood of RV infected children is not associated with PBMC, and their PBMC do not express RV NSPs.

The relatively higher infection of B cells and DC that we found, compared to T cells, (Figs. 1 and 2) is consistent with

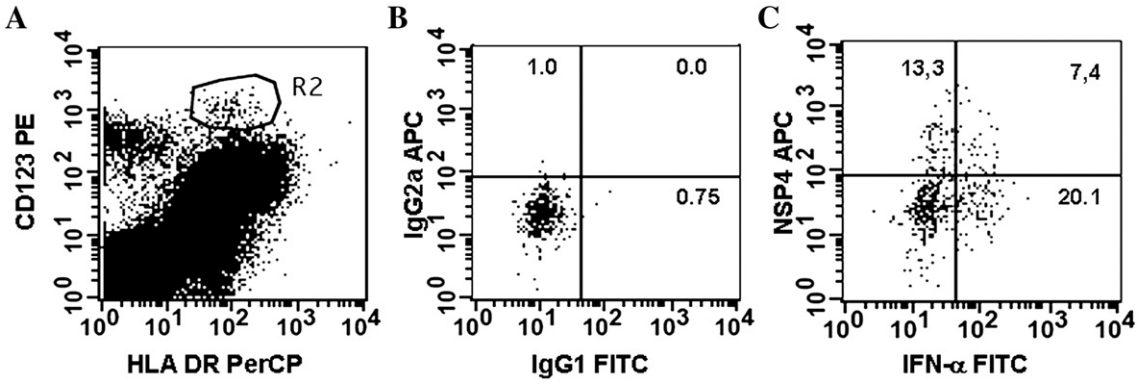

Fig. 6. NSP4+ and NSP4− human pDC secrete IFN-α. PBMC were exposed to purified RRV (MOI=5) for 8 h, in the presence of Brefeldin A in the last 3 h, for simultaneous detection of intracellular NSP4 and IFN-α. (A) pDCs were gated (R2) as HLA-DR^{int}/$CD123^{high}$. (B) Staining of pDC (R2) with isotype control MAbs. (C) Staining of pDC (R2) with antibodies to IFN-α+ and NSP4+. The numbers in the quadrants are the percentage of positive cells. Data are representative of 3 independent similar experiments.

Table 1
Study population

Group	Age [a]	HAOD [b]	Diarrhea [c]	Vomit [d]	Plasma IgA [e]	RV Ag (% of positive Control)			RV P genotype
						Feces	Plasma	PBMC	
I	13	192	7	7	100	0.0	0.0	ND	Neg
I	5	24	3	1	Neg	0.0	0.0	ND	Neg
II	6	72	6	0	Neg	0.9	0.0	0	P[8]
II	12	30	18	15	800	19.0	0.0	ND	P[8]
II	14	144	5	5	Neg	56.7	0.0	ND	P[8]
II	14	240	8	7	1600	87.0	0.0	0	P[8]
II	11	120	15	5	Neg	90.9	0.0	0	P[8]
II	3	16	4	2	Neg	94.5	0.0	ND	P[8]
III	20	11	3	5	Neg	84.5	55.3	ND	P[8]
III	10	30	7	6	Neg	87.3	78.9	ND	P[8]
III	16	48	10	4	Neg	88.9	93.6	0	P[6]
III	16	48	10	8	Neg	92.2	90.0	ND	P[8]
III	18	36	3	5	Neg	94.7	100.6	0	P[4]
III	20	96	1	4	Neg	96.0	85.0	0	P[6]
III	17	48	8	3	Neg	97.0	89.8	0	P[8]
III	18	24	12	12	Neg	97.6	89.3	ND	P[8]
III	11	72	5	4	Neg	115.0	120.0	ND	P[8]

Neg: not detectable. ND: not done.
[a] Age of the children in months.
[b] Hours after onset of diarrhea.
[c] Maximum number of diarrhea episodes per day.
[d] Maximum number of vomit episodes per day.
[e] Reciprocal of IgA titer.

results with murine mesenteric lymph node cells, in which NSP4-positive cells were found expressing either B220 (a B-cell marker also found on pDC), CD11c (a mDC marker) or CD11b (a marker expressed on macrophages, NK cells, granulocytes, and activated lymphocytes), but not in CD3+ T cells (Fenaux et al., 2006). The factors that determine the relative susceptibility to infection of the different subsets of PBMC are unknown. In our previous publication we showed that RV infected higher numbers of mature than immature moDC (Narvaez et al., 2005). Since mature moDC express higher levels of the α4 integrin than immature moDC, and this integrin has been shown to be one of the receptors for RV (Lopez and Arias, 2006), we hypothesized that this could be a possible explanation. Because both B and T cells express on average the same levels of the α4 integrin (Rott et al., 2000), other factors seem necessary to explain their differential susceptibility to infection.

In contrast to our *in vitro* data, RV NSPs could not be detected in PBMC from RV-infected children. This is most likely due to the neutralizing capacity of factors (antibodies, cytokines, etc.) present in serum (Fig. 3), and/or to the low levels of infectious virus circulating in blood (Ray et al., 2006). It may also be that human RVs, like the Wa virus we have evaluated (Fig. 3), have a lower capacity to infect PBMC than RRV that has been shown to have a broad tissue tropism (Crawford et al., 2006). Finally, RV infection of PBMC seems to be transient (Fig. 2) and it is also possible that only abortive infections are present in these cells. However, we can not rule out that low levels of infection are occurring in selected subpopulation of PBMC of infected children. This is particularly true for the pDC, which are present at low levels in the children evaluated.

Of note, cellular viability in the cultures of PBMC treated with RRV (evaluated by trypan blue exclusion) was higher than 90%. However, 24 h post-treatment important reductions in the relative frequencies of monocytes and DC were observed in both the RV and control treated cultures. For this reason, we did not evaluate infection in these subpopulations of PBMC past this time (Fig. 2). This finding is expected due to the known dependence of these cells on specific growth factors for survival. In our previous publication we showed that RRV did not induce cell death of human moDC (Narvaez et al., 2005). From our present results it is difficult to asses if RV is inducing cell death of monocytes and/or DC, and future experiments are needed to determine if this is the case. Studies to establish if RV is performing a complete replicative cycle in any of the PBMC subsets studied will also shed light on the way RV interacts with PBMC. Nevertheless, this seems unlikely since in human moDC (Narvaez et al., 2005) and murine mDC (Douagi et al., 2007) only very low levels or not complete RV replication is seen.

PBMC exposed to RRV initially secreted IL-6, TNF-α and IFN-α but no IL-12, and in a second phase they secrete IFN-γ and small amounts of IL-2 and IL-10 (Fig. 3). This pattern of secreted cytokines is concordant with that detected in the serum of RV infected children (Dubos et al., 2004; Jiang et al., 2003), suggesting that PBMC maybe contributing to the production of these cytokines. Moreover, it suggests the induction of a Th1 type T cells by a pathway dependent on IFN-α and not of IL-12 (Liu, 2005). This interpretation is in agreement with our previous study showing only low levels of secretion of IL-12 by mature human moDC exposed to RRV (Narvaez et al., 2005). As dsRNA activated mDC (but

not pDC (Ito et al., 2006)) are the principal source of IL-12 in viral infections, the absence of this cytokine suggests that RV dsRNA is not available to stimulate TLR-3 in immature mDC (Matsumoto et al., 2003). However, these findings contrast with results in which cultures of simian moDC treated with RV and T cells secrete IL-12 (Sestak et al., 2004). Thus, under certain circumstances RV may stimulate an IL-12 milieu that could induce Th1 T cells.

The high levels of IFN-α we observed in the cultures of PBMC exposed to RRV is somewhat at odds with studies that have shown that in the intestinal epithelial cell line, Caco-2, RV NSP1 can induce degradation of IFN regulatory factor 3 and 7, which are key to produce IFN-α (Barro and Patton, 2005, 2007). Nonetheless, they are consistent with recent results in the mouse model where mDC and pDC, but not fibroblasts, exposed to RV secrete IFN-α (Douagi et al., 2007). Thus, the inhibitory effect of NSP1 on the production of IFN-α seems to be less efficient in DC infected with RV. However, as shown in the murine model, NSP1 could be down-regulating some production of IFN-α in DC (Douagi et al., 2007). We observed that a group of non-infected pDC produced IFN-α and that only a minority of infected pDC were secreting IFN-α (Fig. 5). This result suggests that some of the IFN-α secreting pDC maybe activated by bystander mechanisms, and that RV may have the capacity to inhibit IFN-α in a subset of infected pDC.

The quantity of IFN-γ (Fig. 5) and the frequency of RV specific T cells (Fig. 7) secreting IFN-γ were diminished in cultures of PBMC depleted of pDC, indicating a role for pDC in stimulating RV-specific memory T cells. In agreement with this finding, it has been shown that pDC can directly stimulate memory T cells specific for influenza virus (Fonteneau et al., 2003). On the other hand, pDC could also be fulfilling an indirect role in stimulating the T cell by "helping" mDC to present viral antigen, as has been shown in the mouse model for HSV (Yoneyama et al., 2005) or in a murine model of tumor immunity (Lou et al., 2007). Of note, the involvement of pDC in RV antigen presentation would suggest that they may induce naive T cells to become IL-10-secreting regulatory T cells (Ito et al., 2007), and/or favor the re-stimulation of memory T cells that also secrete this cytokine (Janke et al., 2006). In spite of this, we have not evidenced RV-specific T cells that secrete IL-10, in our short term (10 h) re-stimulation protocol of PBMC (Narvaez et al., 2005). It remains to be determined if the low levels of IL-10 produced late in cultures of RV stimulated PBMC (Fig. 4) are produced by regulatory T cells.

In conclusion, we have performed studies of the interaction of RV with PBMC cells that may potentially shed light on characteristics of the systemic anti-RV immune response. The lack of infection of PBMC from RV infected children with antigenemia suggests that infectious RV is not the principal antigen being presented systemically to T cells. This may be an important factor that determines the repertoire of systemic RV-specific T cells. Future studies are necessary to establish the relevance of our results suggesting a role for pDC in stimulating RV-specific memory T cells to the *in vivo* situation in children.

Materials and methods

Subjects and sample collection

Fourteen healthy adults (21–42 years old), and 17 children (2–24 months old) admitted with gastroenteritis to the pediatric emergency service of the San Ignacio Hospital in Bogotá (Colombia) were enrolled in this study. All adults and children's parents/guardians signed an informed consent approved by the Ethics Committee of the Medical School of the Pontificia Universidad Javeriana. Blood samples were drawn from volunteers to obtain serum and/or plasma and PBMC. Stool samples were collected only from children, and kept at −70 °C until use. PBMC were isolated by density gradient using Lymphosep medium ($d=1.077$) (ICN Biomedical Inc, Irvine, CA). The cells were then washed, resuspended in AIM-V media (Gibco, Grand Island, NY) and used immediately. For some experiments, we added 150 μl of PBS to a pellet of $3–5\times10^6$ PBMC isolated from children and cells were lysed by three freeze–thaw cycles (PBMC lysate). These lysates were centrifuged at $16{,}000\times g$ for 5 min at 4 °C and supernatants stored at −70 °C until analysis.

Viruses and cells

The RRV strain of simian RV (obtained from Dr. H. B. Greenberg, Stanford University CA) was treated with 2 μg/ml trypsin (Sigma, St. Louis, MO) for 15 min at 37 °C and then grown in MA104 cells in the presence of 1 μg/ml trypsin for 48 h at 37 °C (Jaimes et al., 2002). Cells were lysed by repeated freezing and thawing, and RV was purified by ultracentrifugation in CsCl gradient (Jaimes et al., 2002). The purified stock virus preparations had titters between 2.5×10^8 and 1×10^9 focus forming units (ffu)/ml. Before use, the virus was dialyzed against RPMI and a fraction of this dialyzing media was saved to be used as control (Mock control). In some experiments, RRV was used as a clarified tissue culture supernatant and the supernatant of Mock-infected MA104 cells was used as a control for these preparations. The human Wa strain (G1P[8]) RV (obtained from Dr. E Mendez, UNAM Mexico) was similarly grown in MA104, and semi-purified on a 40% sucrose gradient. The Wa stocks used had titters between 2 and 3×10^8 ffu/ml. Supernatants of MA104 cells cultured without Wa and treated similarly were used as Mock controls. The RNA patterns of both RV strains were confirmed by polyacrylamide gel electrophoresis.

RV infection of PBMC

PBMC (25×10^6 cells) were exposed to RV (MOI=5) and its respective Mock control for 30–45 min at 37 °C with 5% CO_2. Cells were then resuspended in 5 ml AIM-V medium (Gibco) and distributed in 1 ml aliquotes (5×10^6 PBMC) in 15 ml polypropylene tubes. Aliquots were incubated with a 5° angle inclination at 37 °C with 5% CO_2 for different periods of time. In some experiments, 5×10^6 PBMC were stimulated with

2.5 μg/ml *Staphylococcus aureus* enterotoxin type B (SEB, Sigma), or 0.038 μM 2395 CpG (Coley Pharmaceutical Group Inc, Wellesley, MA), as positive controls. After incubation, cellular suspensions were centrifuged; supernatants were collected and kept at −70 °C for cytokine evaluation, and cells were stained for flow cytometry analysis. In some experiments, PBMC were infected in the presence of 10% autologous decomplemented (30 min 56 °C) serum, RV-neutralizing anti-VP7 MAbs (159 or 2C9 ascites, G3 and G1 specific, respectively) supplied by Harry B. Greenberg or IgG1κ isotype control MAb (BD Bioscience, San Diego, CA).

Detection of intracellular NSP4 and NSP2 in PBMC

To establish if PBMC are permissive to RV infection, the cells exposed to RV or Mock preparations were stained for intracellular detection of NSP4, as previously described (Narvaez et al., 2005), with some modifications. Cells were resuspended in PBS–0.05 mM EDTA for 10 min and then washed with staining buffer [0.5% BSA (Merck KGaA, Darmstadt, Germany), 0.02% sodium azide (Mallinckrodt Chemicals, Paris, KY) in PBS], fixed with 1% paraformaldehyde (Electron Microscopic Sciences, Hatfield, PA) for 5 min, washed and permeabilized with 1× permeabilizing solution (BD Bioscience) for 10 min at room temperature (RT). Then, the cells were washed and FcγRs (receptors present on PBMC and responsible for non-specific binding of immunoglobulins to cells) were blocked with 30% autologous serum, for 10 min at RT. Afterwards, cells were incubated with anti-NSP4 MAb (B4-2) or, anti-NSP2 MAb (191) (both supplied by Harry B. Greenberg) or their respective IgG2a and IgG1 isoytpe controls (BD Biosciences) for 30 min at 4 °C. After washing the cells, FCγRs were blocked again with 30% of a 9:1 mixture of normal goat serum (Vector): autologus serum, for 10 min at RT. Then, allophycocyanin labeled goat anti-mouse immunoglobulin (GAM-APC, Invitrogen Molecular Probes, Eugene, OR) was added for 30 min at 4 °C. Finally, cells were stained for 30 min at 4 °C with monoclonal antibodies specific for the different cell subsets (all from BD Biosciences unless otherwise noted): 1) monocytes: anti-CD14FITC, B cells: anti-CD19PE and T cells: anti-CD3PerCP; 2) NK cells: NK cocktail (anti-CD3FITC/anti-CD16PE/anti-CD56PE/anti-CD45PerCP); 3) mDC: Lin-1FITC (anti-CD3/anti-CD14/anti-CD16/anti-CD56/anti-CD19/anti-CD20)/anti-CD11cPE/anti-HLA-DRPerCP; and 4) pDC: Lin-1FITC/anti-CD123PE/anti-HLA-DRPerCP. In some experiments anti-CD19FITC/anti-CD16PE/anti-CD56PE/anti-CD3PerCP or anti-CD3FITC/anti-CD16PE/anti-CD56PE/anti-CD20PECy5 (Dako) were used. Then, cells were washed with staining buffer, resuspended in 1% paraformaldehyde in PBS, acquired on a FACSCalibur (BD Bioscience) equipped with a second 635-nm red diode laser, and analyzed with Cellquest Pro software. Dead cells and debris were excluded by forward and side scatter gating. Results are expressed as net values: % NSP4+ cells in PBMC treated with RRV minus % NSP4+ cells in PBMC treated with the Mock preparation.

PBMC from RV-infected children were stained as described above, but blocking FcγRs with autologous serum, and staining NK cells with anti-CD56PE/anti-CD16PE/anti-CD3PerCP (all from BD Biosciences). The data for RV NSP4 or NSP2 expression was analyzed only when the number of cells for a given subset was higher than 100.

Detection of cytokines in cell culture supernatants

Frozen supernatants from PBMC exposed to RRV, the Mock preparation, 2.5 μg/ml SEB or 0.038 μM CpG 2395 were thawed to measure cytokines according to manufacturer's instructions. IL-12p70, TNF-α, IL-10, IL-6, IL-1β and IL-8 were evaluated using the cytometric bead array (CBA) inflammation kit (BD Biosciences) and IFN-γ, IL-4, IL-5 and IL-2 with the Th1/Th2 CBA kit (BD Biosciences). The CBAs detected between 1.9 and 7.2 pg/ml of cytokines. ELISA kits were used for quantifying TGF-β (Duoset, R&D systems, Minneapolis, MN) and IFN-α (PBL Biomedical Laboratories, Piscataway, NJ), following the manufacturer's instructions. The assays detected 31.2 pg/ml of TFG-β and 10 pg/ml of IFN-α. Results are expressed as net values: cytokine level in PBMC exposed to RRV minus cytokine level in PBMC exposed to Mock. For some experiments PBMC were depleted of pDC with the BDCA-2 isolation kit (Miltenyi Biotec Inc, Auburn, CA) following the manufacturer's instructions. As control experiments PBMC were treated with GAM beads (Myltenyi) or depleted of B cells using anti-CD19 immunomagnetic microbeads (Miltenyi). Depletion of B cells or pDC was confirmed by staining the resultant population with anti-CD19PE and Lin-1FITC/anti-HLA-DRPerCP/anti-CD123PE, respectively. In both cases the cell subset reductions were higher than 85%.

Simultaneous detection of viral NSP4 and IFN-α in pDC

PBMC treated with RRV or Mock control were incubated for 8 h at 37 °C. To improve detection of IFNα, 10 μg/ml Brefeldin A (Sigma) was added during the last 3 h (Dai et al., 2004; Krug et al., 2001). Then, cells were washed, fixed, permeabilized and stained as described above for detection of NSP4. Cells were washed, resuspended in staining buffer and incubated overnight at 4 °C. The next day, cells were washed with cold 5% fetal calf serum (FCS, Gibco-BRL) in PBS, blocked with 10 μl FcγR blocking reagent (Miltenyi) for 10 min at RT and incubated with 0.38 μg MAb anti-IFN-αFITC (PBL) or IgG1FITC isotype control (BD Biosciences) for 30 min at 4 °C. Then, cells were washed and incubated 30 min at 4 °C with anti-CD123PE and anti-HLA-DRPerCP. Finally, cells were washed, fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometry.

Detection of circulating memory IFN-γ+ RV specific T cells

Frequencies of circulating memory RV-specific CD4+ and CD8+ cells were determined as previously described (Jaimes et al., 2002). Briefly, 2×10^6/2 ml total PBMC, pDC cells-depleted PBMC, or B-depleted PBMC were incubated with RRV lysate (MOI=5) or Mock lysate, in the presence of anti-CD49d MAb 0.5 μg/ml (BD Biosciences) and anti-CD28 MAb 0.5 μg/ml

(BD Biosciences) for 10 h at 37 °C. Brefeldin A (10 μg/ml) was added after 5 h of incubation. Cells then were washed, fixed and permeabilized as previously described, and incubated with MAbs anti-IFN-γFITC or IgG1FITC isotype control, anti-CD69PE, anti-CD4PerCP and anti-CD8APC (all from BD Biosciences), for 30 min at 4 °C. Cells were then washed, resuspended in 1% paraformaldehyde and analyzed by flow cytometry. CD69 is a marker of recently activated T cells and is used to identify recently *in vitro* activated memory T cells (Jaimes et al., 2002). Results are expressed as the net percentages of CD69+/CD4+ or CD69+/CD8+ cells expressing IFN-γ after subtracting values observed in the Mock control treated PBMC.

ELISAs for measuring RV antigen and RV specific IgA

The presence of RV antigen in feces was determined by using an enzyme-linked immunosorbent assay (ELISA) previously described (Jaimes et al., 2002). A supernatant from RF virus-infected MA104 cells was used as a positive control and a known RV negative human fecal specimen was used as a negative control in each plate. Results are expressed as percentage of reactivity referred to the positive control. For detection of RV antigen in plasma and PBMC, the same protocol described was followed, with minor modifications: plasma samples were diluted in Blotto 2.5% and non diluted PBMC lysates were used (Crawford et al., 2006), and three instead of five washes were made.

RV-specific IgA in plasma were detected by ELISA, as previously described (Gonzalez et al., 2003).

Nucleic acid extraction and P typing RT-PCR

All stool samples were used for nucleic acid extraction and P genotyping. Double-stranded RNA (dsRNA) was extracted from 10% stool suspensions in Phosphate-Buffered Saline with a QIA Amp Viral RNA Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. P typing employed nested, multiplex RT-PCR and used consensus and type-specific primers described previously (Gentsch et al., 1992). Consensus primers Con2 and Con3 were used in a first round RT-PCR (40 cycles) to generate a 876-bp fragment of gene 4; Con3 was then used in a second round PCR (40 cycles) with types-specific primers P[8] (1-T1), (VP4NAC10), (VP4NAC25), P[4] (2-T1) and P[6] (3-T1). The amplified products were detected by electrophoresis on a 2% agarose gel containing ethidium bromide (0.5 μg/ml), and visualized under ultra violet illumination (Gentsch et al., 1992).

Statistical analysis

Statistical analysis was performed with Prisma software version 3.02, using non-parametric tests. Differences between groups were evaluated by Mann–Whitney tests. Differences between paired results within a study group were compared with the Wilcoxon test. Correlations between groups were analyzed with Spearman's test. Significance was established if $p < 0.05$. Data are shown as median, range, unless otherwise noted.

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